2. Background

FINDING TRACES OF MICROBIAL METABOLISMS IN THE ROCK RECORD

Microbial metabolisms contribute to the maintenance of the hydro-, atmo-, and lithospheres on the Earth and have done so since they first evolved on this planet billions of years ago [98, 114, 126]. While determining the impact of microbes in modern environments is a tractable problem, given that the activities of these organisms can be monitored directly *in situ* or in pure culture [21, 115, 129, 135], investigating the impact microbes had on the chemistry of the environment billions of years ago presents a formidable challenge.

When the organisms are macroscopic, relationships between biology and the geochemical evolution of the Earth can be inferred from morphological fossils [100]. While this approach can also be applied to microorganisms, when considering the impact microbes have had during remote periods of Earth history (*e.g.,* the Archean, >2.5 Ga), the fossil record of these organisms becomes increasingly poor as the rocks we look at increase in age and even when microfossils are found, they can be highly controversial [23, 148]. Moreover, these fossils provide little evidence regarding the physiology of the organisms they represent.

An alternate, accepted approach to recognizing biological activity in the ancient rock record is to identify organic or inorganic signatures unique to extant

microbial metabolisms that are preserved in rocks through time (*i.e.,* biosignatures). When biosignatures indicative of a particular metabolism are identified, inferences regarding the impact the metabolism may have had on the environment can be made [31, 143, 153, 166, 171]. And while we can never know the extent to which modern metabolisms are good proxies for ancient ones, because we cannot study extinct organisms, the assumption that they are is accepted as a necessary one in this field [3].

It is important to note that, just like morphological fossils, biosignatures are subject to controversy and misinterpretation [121, 147, 175]. Thus, identifying robust biosignatures unique to a particular metabolism that cannot be confused with abiotic processes represents a true challenge. Nonetheless, the identification of such signatures is a necessary first step towards understanding how microbial metabolisms have influenced the chemistry of the Earth over time.

To make inferences about the cycling of elements on the ancient Earth, it is important to identify biosignatures of organisms that carry out an ancient form of metabolism. A particular metabolism that has had a profound impact on the chemical evolution of the Earth and that is believed be among the first metabolisms to have evolved is photosynthesis. The antiquity of the oxygenic form of this metabolism is supported by the finding of 2-methylhopane hydrocarbon derivatives of cyanobacterial membrane lipids in rocks as old as 2.7 Ga [25, 166].

Further, phylogenetic relationships between genes that are involved in bacteriochlorophyll and chlorophyll biosynthesis show that the anoxygenic form

of photosynthesis evolved before the oxygenic form [185]. If true, the evolution of anoxygenic photosynthesis would logically predate the evolution of respiratory metabolisms that are based on oxygen or other highly oxidized species (*i.e.,* nitrate) as well. Therefore, the metabolism of anoxygenic photoautotrophic bacteria is of primary interest in our considerations of the geochemical evolution of the Earth.

FE(II) OXIDATION BY PHOTOAUTOTROPHIC BACTERIA

Microbial Fe(II) oxidation is an important component of the Fe geochemical cycle [125, 158, 160]. In modern environments, microorganisms that are able to oxidize Fe(II) are ubiquitous, inhabiting and affecting a wide variety of environments where Fe(II) is present. These environments include: marine coastal sediments and brackish water lagoons [161, 163], sediments from freshwater creeks, ponds, lakes and ditches [68-70, 182], low pH environments associated with acid mine waters [39, 50], groundwater springs [54], sediments and the rhizosphere of plants from freshwater wetlands [56, 157], the seafloor near active hydrothermal fields [51, 55], and swine waste lagoons [34].

Microorganisms that are able to oxidize Fe(II) are diverse in their phylogeny and overall physiology (Table 2-1). Representative examples of bacteria and archaea capable of coupling Fe(II) oxidation to growth include psychro-, hyperthermo- and mesophiles that couple Fe(II) oxidation to the reduction of nitrate at neutral pH [14, 162], or to the reduction of oxygen at either

low [50, 169], or neutral pH [54], and the anaerobic Fe(II)-oxidizing phototrophs [52, 70, 182].

Table 2-1: Metabolisms where Fe(II) is the electron donor and the genes that have been implicated in these processes.

Metabolism	Reaction	Genes
Acidophilic iron oxidation	$4Fe^{2+} + 4H^{+} + O_2 \rightarrow 4Fe^{3+} + 2H_2O$	*iro, cyc1, cyc2, coxA,B,C,D, rus*
Phototrophic iron oxidation	$4\ Fe^{2+} + HCO_3^{-} + 10H_2O \rightarrow 4\ Fe(OH)_3 + (CH_2O) + 7H^{+}$	None known
Neutrophilic iron oxidation	$4Fe^{2+} + 10H_2O + O_2 \rightarrow 4Fe(OH)_3 + 8H^{+}$	None known
Nitrate-dependent iron oxidation	$10\ Fe^{2+} + 2\ NO_3^{-} + 24\ H_2O \rightarrow 10\ Fe(OH)_3 + N_2 + 18H^{+}$	None known

The use of Fe(II) as an electron donor likely arose early in Earth history given the abundant availability of Fe(II) in the ancient oceans, relative to today [57, 72, 184] and of the organisms able to grow on Fe(II), it is thought that the anoxygenic phototrophs are the most ancient [20, 41, 185]. Thus, in addition to contributing to Fe cycling in modern environments, Fe(II)-oxidizing bacteria have likely affected the Fe cycle over geological time. Indeed, both direct photoautotrophic Fe(II) oxidation and indirect Fe(II) oxidation mediated by cyanobacteria [37] have been proposed as being responsible for the deposition of Banded Iron Formations as discussed in the introduction [67, 101, 182]. To distinguish these two biological processes from each other, as well as from other

proposed abiotic mechanisms of Fe(II) oxidation, biosignatures that uniquely represent the activity of Fe(II)-oxidizing organisms must be identified. An informed search for such biosignatures and their rigorous interpretation requires a detailed understanding of the mechanism and products of Fe(II) photoautotrophy. To date, very little is known about the mechanism of this metabolism. Therefore, our studies have been focused on elucidating the molecular basis of Fe(II) oxidation by these bacteria.

MECHANISMS OF FE(II) OXIDATION BY ACIDITHIOBACILLUS FERROOXIDANS

Although little is known about Fe(II) oxidation in phototrophic bacteria at the mechanistic level, a substantial body of knowledge concerning the mechanism of this metabolism exists for the acidophilic, Fe(II)-oxidizing organism, *Acidithiobacillus ferrooxidans*. Members of this species are gram-negative, mesophilic, obligately autotrophic and acidophilic bacteria capable of aerobic respiration on Fe(II) and reduced forms of sulfur (H_2S, S^o, $S_2O_3^{2-}$) [53, 139]. Because they can grow chemolithoautotrophically on sulfide ores, these bacteria are able to solubilize a variety of valuable metals such as copper, uranium, cobalt, and gold that are embedded within the ores [138]. Given this property, understanding the metabolism of these bacteria is particularly interesting to industries wishing to use this strain (or genetically modified derivatives) for leaching purposes [7, 137, 183].

Most of what is known about Fe(II) oxidation by *A. ferrooxidans* stems from biochemical studies, yet how the different components of the electron transport pathway (Figure 2-1) work together is uncertain and controversial [7, 19, 77, 186]. Comparatively little is known about the genetics of Fe(II) oxidation in *A. ferrooxidans* as genetic analysis has been constrained by the culturing requirements for this organism. For example, a number of antibiotics are inhibited by low pH and high Fe(II) concentrations [183], resulting in a dearth of suitable selective markers. To circumvent this problem, toxic metal resistance genes have been used as selective markers, but only with limited success [103]. Additionally, while some of the standard tools required for genetic studies (*e.g.,* appropriate shuttle vectors and transformation methods) have been developed and/or optimized for various strains of *A. ferrooxidans* [103, 130, 138], until recently [109], these methods have not been used for the construction of mutants. Consequently, no defined mutants defective in Fe(II) oxidation exist, although spontaneous mutants that have lost the ability to oxidize Fe(II) have been identified [149]. The recent report of the construction of a *recA* mutant of *A. ferrooxidans* strain ATCC 33020 via marker exchange mutagenesis represents a step towards improved genetic analysis of this strain [109].

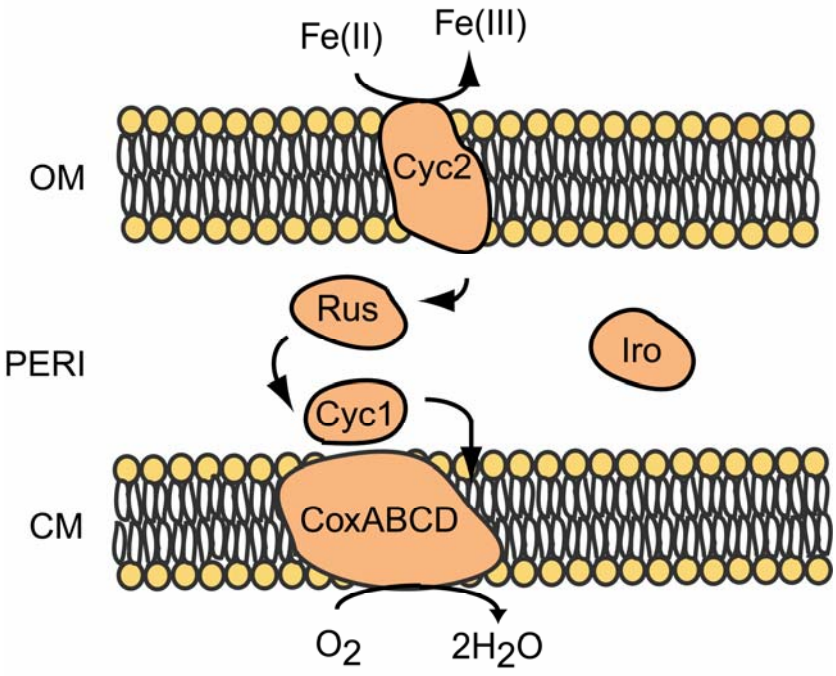

Figure 2-1: Cartoon representation of the components implicated in electron transfer for Fe(II) oxidation by *Acidithiobacillus ferrooxidans* strain ATCC 33020. The product of the *iro* gene is not thought to play a role in this strain, but may in others.

Despite these limitations, several genes thought to be involved in Fe(II) oxidation are known. The majority have been identified using degenerate primers derived from N-terminal sequences of purified proteins. The first of these genes to be identified using reverse genetics was the *iro* gene of *A. ferrooxidans* strain Fe1. This gene encodes a high potential Fe-S protein that is homologous to the soluble ferredoxins commonly found in purple photosynthetic bacteria. Additionally, Northern blot and RNA primer extension analyses suggest that this gene is transcribed on its own, but expression studies under different growth conditions have not yet been conducted [104]. Because of its high redox

potential, Fe(II)-cytochrome *c*-552 oxidoreductase activity and acid stability *in vitro,* it has been proposed that the product of this gene catalyzes the first step in the transfer of electrons from Fe(II) to O_2 [63, 104, 187]. However, this does not appear to be the case for all strains of *A. ferrooxidans* [7] and genetic evidence to support this function in *A. ferrooxidans* Fe1 does not exist.

A second gene thought to encode a protein involved in Fe(II)-oxidation by *A. ferrooxidans* is the *rus* gene. Again, using reverse genetics, the *rus* gene was cloned from *A. ferrooxidans* ATCC 33020 [13, 66]. This gene encodes the small type 1 blue copper protein, rusticyanin; a protein that has received much attention in biochemical studies given that it represents up to 5% of the total soluble protein of *A. ferrooxidans* cells when grown on Fe(II), displays a high degree of acid tolerance and has a high redox potential [40, 76]. In the region upstream of the *rus* gene, a sequence similar to a rho-independent terminator and two potential *Escherichia coli*-like, σ^{70}-specific promoter sequences are present. Downstream of the gene are two putative stem loop structures, one of which is followed by a T rich region. This suggests that the *rus* gene can be transcribed from its own promoter [13]. Further investigations of *rus* gene transcription by Northern, RT-PCR and primer extension analyses have shown that this gene is part of an operon comprising eight genes, of which *rus* is the last [7, 13]. Putative promoters in this operon have been identified both by sequence and primer extension analyses. Primer analysis with RNA extracted from cells grown on sulfur or Fe(II) indicates that two promoters upstream of *cyc2* and one promoter upstream of *rus* are active in cells grown on sulfur whereas only one of

the promoters upstream of *cyc2* is active in Fe(II)-grown cells [7]. Additionally, while it has been observed that the *rus* transcript is present in both Fe(II) and sulfur grown cells, it is more abundant in Fe(II)-grown cells and present throughout all growth phases (in contrast to sulfur-grown cells, where it appears only in exponential phase) [188, 191].

Since the discovery that *rus* is co-transcribed with several other genes in an operon (Figure 2-2), the genes in this operon have been analyzed [6, 7, 190]. Strikingly, seven of the eight genes in this operon appear to encode redox proteins. The *cyc2* gene encodes a high molecular weight, outer membrane, *c*-type cytochrome [6, 190] while *cyc1* encodes a c_4-type cytochrome with a signal peptide sequence indicative of translocation to the periplasm [6]. *coxB*, *coxA,* and *coxC* encode proteins with homology to subunits II, I, and III, respectively, of an aa_3-type cytochrome *c* oxidase. The protein encoded by *coxD* shares similarity with nothing in the database, however, given the position of this gene relative to the other *cox* gene homologs and what is known about the organization of these genes in other organisms, it was assumed that *coxD* represents subunit IV of an aa_3-type cytochrome *c* oxidase [7]. Lastly, ORF1 encodes a putative protein of unknown function with a terminal signal sequence suggesting that it is translocated to the periplasm. Given that biochemical studies have implicated proteins of these types in Fe(II) oxidation [17, 38, 40, 63, 76, 174, 177], it is assumed that the products of this operon are involved in the Fe(II) respiratory pathway of this organism (Figure 2-1) [7].

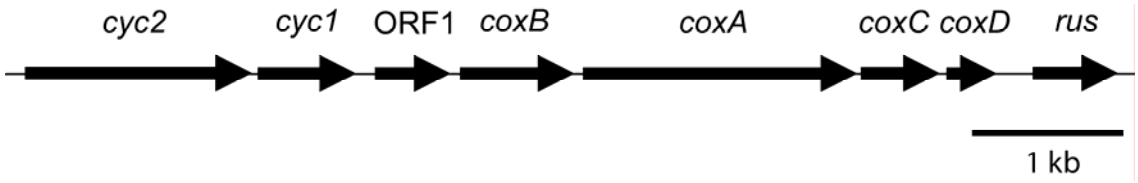

Figure 2-2: Genes proposed to encode the components of Fe(II) oxidation in *Acidithiobacillus ferrooxidans* strain ATCC 33020.